Integration of Global Regulation of Two Aromatic-Responsive σ^{54} -Dependent Systems: a Common Phenotype by Different Mechanisms

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Pseudomonas-derived regulators DmpR and XylR are structurally and mechanistically related σ^{54} -dependent activators that control transcription of genes involved in catabolism of aromatic compounds. The binding of distinct sets of aromatic effectors to these regulatory proteins results in release of a repressive interdomain interaction and consequently allows the activators to promote transcription from their cognate target promoters. The DmpR-controlled Po promoter region and the XylR-controlled Pu promoter region are also similar, although homology is limited to three discrete DNA signatures for binding σ^{54} RNA polymerase, the integration host factor, and the regulator. These common properties allow cross-regulation of Pu and Po by DmpR and XylR in response to appropriate aromatic effectors. In vivo, transcription of both the DmpR/Po and XylR/Pu regulatory circuits is subject to dominant global regulation, which results in repression of transcription during growth in rich media. Here, we comparatively assess the contribution of (p)ppGpp, the FtsH protease, and a component of an alternative phosphoenolpyruvate-sugar phosphotransferase system, which have been independently implicated in mediating this level of regulation. Further, by exploiting the cross-regulatory abilities of these two circuits, we identify the target component(s) that are intercepted in each case. The results show that (i) contrary to previous speculation, FtsH is not universally required for transcription of σ^{54} -dependent systems; (ii) the two factors found to impact the XylR/Pu regulatory circuit do not intercept the DmpR/Po circuit; and (iii) (p)ppGpp impacts the DmpR/Po system to a greater extent than the XylR/Pu system in both the native *Pseudomonas putida* and a heterologous *Escherichia coli* host. The data demonstrate that, despite the similarities of the specific regulatory circuits, the host global regulatory network latches onto and dominates over these specific circuits by exploiting their different properties. The mechanistic implications of how each of the host factors exerts its action are discussed.

Soil microorganisms such as *Pseudomonas putida* have the ability to aerobically catabolize a wide range of aromatic hydrocarbons via suites of specialized catabolic enzymes (reviewed in references 36 and 72). Operons encoding these catabolic enzymes are frequently found on plasmids or, if located on the chromosome, are often carried on transposons or flanked by insertion sequences, ensuring a degree of transferability (72). The ability to catabolize aromatics is nonessential, serving only to confer an advantage to the host bacterium under conditions where aromatic compounds are readily available. Therefore, retention of these catabolic systems is only truly advantageous if their usefulness does not compromise the host fitness under other conditions. Thus, aromatic catabolic systems have been found to be tightly regulated in response to signals specific to their specialized function and also to be appropriately subservient to the existing evolutionarily adapted host global regulatory network (2, 17, 25, 31, 42, 68). However, the underlying mechanisms and the dominant global regulatory systems involved have only been addressed in a few cases. Among these are the pVI150-borne *dmp* system of *Pseudomonas* sp. strain CF600, which mediates conversion of (methyl)phenols to pyruvate and acetyl-coenzyme A (reviewed in ref-

erence 54), and the pWW0-borne *xyl* upper operon, which encodes enzymes for conversion of toluenes and xylenes into benzoate intermediates, which are further transformed by enzymes of the *xyl* lower (*meta*) pathway (reviewed in reference 41). Host-imposed regulation is manifested in both systems as repression of transcription during rapid growth in rich media even though the specific activation signal is present (12, 25, 68). Conceptually, the functional outcome is analogous to glucose catabolite repression of the use of alternative sugars such as lactose in enterics, i.e., the silencing of alternative metabolic pathways until preferred nutrients are in short supply (69).

The major regulators of the *dmp* and *xyl* systems, DmpR and XylR, control transcription in response to distinct sets of aromatic effector compounds representing pathway substrates and structural analogues (41, 60, 62). The Po promoter is the sole target for DmpR, while XylR controls both the Pu (upper-operon) promoter and one of the promoters of the Ps region controlling transcription of the *xylS* regulatory gene (41). DmpR and XylR are highly homologous σ^{54} -dependent regulators (35, 60) that have many structural and mechanistic features in common. The N-terminal signal reception domains of the two regulators can be exchanged without affecting common activator function, but this results in a corresponding switching of aromatic effector profiles (62, 65). Furthermore, independent genetic and biochemical studies have led to the conclusion that the two regulators are activated upon aromatic effector binding by similar derepression mechanisms involving

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FIG. 1. (A) Regulatory elements of *dmp* and *xyl* systems, as described in the text. The *dmpR* gene is organized in a divergent configuration from its target promoter *Po*. The *xylR* gene is similarly orientated relative to *Ps*, the upstream activating sequences (UASs) of which overlap with the *xylR* promoter, while its other target promoter, *Pu*, is located distally on the pWW0 plasmid. Indicated *Pvu*II and *Xmn*I restriction sites were used as working boundaries for *P_{dmpR}* and *P_{xylR}*, respectively, in instances where the regulatory genes have to be dissected from their divergently positioned promoters. (B) Schematic representation of the chromosomal inserts in KT2440::*dmpR* and KT2440::*xylR* derivatives. (C) Schematic representation of reporter constructs (*lacZ* and *luxAB*) used in this study. White boxes and arrows, regulatory elements that originate from the *dmp* and *xyl* systems, as indicated.

release of repressive interdomain interactions that constrain the intrinsic ATPase activity and transcriptional activating property of the regulators (28, 47–49, 52, 62). Conformational changes induced by the released ATPase activities ultimately lead to removal of the repressive effect of σ^{54} on the DNA-melting potential of the RNA polymerase and thus transcriptional initiation (7, 37, 70).

Unlike the DmpR and XylR regulators, which have homology throughout their lengths, the *Po* and *Pu* regions have similarities in only three regions. These include the –24, –12 promoter motif recognized by σ^{54} RNA polymerase ($E\sigma^{54}$), an integration host factor (IHF) binding site required for optimal promoter output, and related upstream activating sequences (UAS) for binding the regulators (Fig. 1A) (1, 20, 64, 67). Although the sequences of the *Po* and *Pu* promoter regions differ outside these regions, the regulator binding motifs are sufficiently similar to allow DmpR binding to *Pu* (53). Moreover, DmpR and XylR can efficiently cross-regulate each other's target promoter in a manner that maintains the dominant physiological control observed in their native systems (29).

The minimal components of the *dmp* and *xyl* systems needed as targets for host-imposed regulation are, in both cases, the regulator and its cognate promoter (12, 34, 68). Studies aimed at identifying global regulatory systems responsible for host-imposed response to cellular physiology have identified differ-

ent players. For the DmpR/*Po* system, a link to cell physiology via stringent response factor (p)ppGpp has been reported (69), while ATP-dependent physiological protease FtsH has been demonstrated to be required for XylR-mediated *Pu* transcription (8). In addition, *ptsN*, a component of an alternative phosphoenolpyruvate-sugar phosphotransferase system (PTS), has been reported to be responsible for glucose repression of the XylR/*Pu* regulatory circuit (11, 14). The similarities of the DmpR/*Po* and XylR/*Pu* systems suggest that global players affecting one system might similarly affect the other. In this work we sought to directly compare the effects of the different global factors on the two related aromatic-responsive systems. In particular, we were interested to determine (i) if the two factors (FtsH and PtsN) previously found to impact the XylR/*Pu* system also interact with the DmpR/*Po* system and (ii) if the role of alarmone (p)ppGpp, previously analyzed predominantly in *Escherichia coli*, affects the two systems to the same extent in the native *P. putida* host. The results show that, despite the similarities in the specific regulation of the *dmp* and *xyl* upper operons, their interactions with the host global regulatory network vastly differ. Furthermore, results obtained by dissecting the contributions of regulators and promoters to the different responses provide evidence that global regulatory systems intercept these specific regulatory circuits by predominantly exploiting properties of the regulators.

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Relevant properties	Source or reference
<i>P. putida</i> strains		
<i>Pseudomonas</i> sp. strain CF600	Hg ^r , prototroph, carrying (methyl)phenol catabolic pVI150	61
KT2440 (pWW0::Tn401)	Res ⁻ prototroph carrying Cb ^r derivative of TOL pWW0	M. Bagdasarian
KT2440:: <i>dmpR</i> -Km,	Chromosomally located <i>dmpR</i> transcribed from its native promoter	60, this study
KT2440:: <i>dmpR</i> -Tel		
KT2440:: <i>xylR</i> -Km,	Chromosomally located <i>xylR</i> transcribed from its native promoter	This study,
KT2440:: <i>xylR</i> -Tel		this study
PP1- <i>dmpR</i>	KT2440:: <i>dmpR</i> -Tel <i>relA</i> ::Km <i>spoT</i> ::Gm, (p)ppGpp ⁰	This study
PP1- <i>xylR</i>	KT2440:: <i>xylR</i> -Tel <i>relA</i> ::Km <i>spoT</i> ::Gm, (p)ppGpp ⁰	This study
<i>E. coli</i> strains		
MG1655Δ <i>lac</i>	F ⁻ λ ⁻ K-12 prototroph, Δ <i>lacX74</i>	This study
CF1693Δ <i>lac</i>	MG1655 Δ <i>lacX74</i> Δ <i>relA251</i> ::Km Δ <i>spoT207</i> ::Cm	This study
A8514	W3110 [IN(<i>rmD-rmE</i>)1] <i>lac</i>	8
A8926	W3110 <i>sfhC220</i> ::Tn10, Δ <i>ftsH3</i> ::Km <i>lac</i>	8
S17λpir	Tp ^r /Sm ^r /Res ⁻ RP4- <i>mob</i> ⁺ ; λpir lysogen, permissive donor host for R6K-based suicide plasmids	22
Vectors		
pBluescript SK	Cb ^r multipurpose cloning vector	Stratagene
pGP704-L	Cb ^r , R6K-based cloning vector	50
pMMB66EH	Cb ^r , broad-host-range RSF1010 <i>lacI</i> ^q -P _{lac} expression vector	30
pRK2501-E	Tc ^r Km ^r , broad-host-range RK2-based cloning vector	39
pRW2A	Tc ^r , broad-host-range RK2-based vector with a promoter-less <i>lacZ</i> gene	39
p34S-Gm	Gm ^r gene flanked by restriction sites	24
p34S-Km3	Km ^r gene flanked by restriction sites	23
pUTminiTn5-Km2	Cb ^r Km ^r , mini-Tn5 suicide donor plasmid	19
pUTminiTn5-Tellurite	Cb ^r Tel ^r , mini-Tn5 suicide donor plasmid	58
pV1397	Cb ^r , pMMB66EH derivative deleted of <i>lac</i> -P _{lac}	62
Expression plasmids		
pMCP1	Sp ^r Sm ^r , pPS10 derivative carrying <i>xylR</i> -Pu- <i>lacZ</i>	8
pTK19	Km ^r , RSF1010 based, carrying <i>xylR</i> transcribed from its native promoter	18
pV1360	Cb ^r , RSF1010 based, carrying Po- <i>luxAB</i>	62
pV1401	Cb ^r , RSF1010 based, carrying <i>dmpR</i> transcribed from its native promoter	62
pV1466	Cb ^r , RSF1010 based, carrying <i>dmpR</i> -Po- <i>luxAB</i>	68
pV1671	Tc ^r , RK2 based, Po controlling transcription of <i>lacZ</i>	This study
pV1672	Tc ^r , RK2 based, Pu controlling transcription of <i>lacZ</i>	This study
pV1673	Tc ^r , RK2 based, Po controlling transcription of <i>luxAB</i>	This study
pV1674	Tc ^r , RK2 based, Pu controlling transcription of <i>luxAB</i>	This study
pV1675	Cb ^r , RSF1010 based, carrying <i>dmpR</i> -Po- <i>lacZ</i> reporter unit	This study
pV1676	Cb ^r , RSF1010 based, carrying <i>xylR</i> -Pu- <i>lacZ</i> reporter unit	This study
pV1677	Cb ^r , RSF1010 based, carrying <i>xylR</i> -Pu- <i>luxAB</i> reporter unit	This study
pV1678	Cb ^r , RSF1010 based, carrying <i>xylR</i> -Po- <i>luxAB</i> reporter unit	This study
pV1679	Cb ^r , RSF1010 based, carrying <i>dmpR</i> -Pu- <i>luxAB</i> reporter unit	This study
pV1680	Cb ^r , RSF1010 based, carrying Pu- <i>luxAB</i> reporter unit	This study
Suicide plasmids		
pV1681	Cb ^r , R6K based, KT2440 <i>relA</i> ::Km on pGP704-L	This study
pV1682	Cb ^r , R6K based, KT2440 <i>spoT</i> ::Gm on pGP704-L	This study

^a Resistance abbreviations: Cb, carbenicillin; Hg, mercuric chloride; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Tel, tellurite; Tp, trimethoprim.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. The schematics of construction of a series of key *dmpR* and *xylR* derivatives used in this study are shown in Fig. 1C. Plasmids were constructed using standard recombinant DNA techniques (57), and DNA generated using the PCR was subjected to sequence analysis. PCR amplification of Po (−233 to +126) and Pu (−205 to +93) promoters as *EcoRI*-*Bam*HI fragments and subsequent insertion into corresponding sites on the *lacZ*-carrying pRW2A gave rise to pVI671 and pVI672, respectively. Plasmids pVI673 (Po, −477 to +2) and pVI674 (Pu, −208 to +2) were likewise constructed by insertion of each of the promoter fragments into a derivative of pRK2501-E carrying the *luxAB* reporter from pVI466. Plasmid pVI675 was derived by replacing the *luxAB* gene on the *Bam*HI fragment of pVI466 with the *lacZ* gene on the *Hind*III fragment of pRW2A with the help of a linker. Replacing the *NotI*-*Bam*HI *dmpR*-Po portion

of pVI675 with the *xylR*-Pu portion from pMCP1 subsequently generated plasmid pVI676. Assembly of an *HpaI*-*XmnI*-bearing *xylR* fragment (from pTK19) and a blunt-ended *KpnI*-*Hind*III-bearing Pu-*luxAB* fragment (from pVI674) onto RSF1010-based vector pVI397 generated pVI677. Plasmid pVI678 was constructed in an analogous manner except that a blunt-ended Po-*luxAB* fragment (*Pvu*II-*Hind*III from pVI466) was used. Plasmid pVI679 was generated using the same pVI397 vector by assembling the *NotI*-*Pvu*II *dmpR* fragment of pVI466 and the *EcoRV*-*Hind*III Pu-*luxAB* fragment of pVI674. RSF1010-based Po-*luxAB* reporter plasmid pVI360 (Po, −477 to +2) has previously been described (62). Plasmid pVI680 was generated to provide an equivalent Pu-*luxAB* reporter plasmid and carries Pu (−208 to +2) as a *SalI*-to-*Bam*HI fragment directly fused to the *Bam*HI *luxAB* fragment.

DNA fragments carrying antibiotic resistance gene replacement of the *relA* and *spoT* homologues of KT2440 were first assembled in pBluescript SK and

then subsequently cloned as *Bam*HI-to-*Xho*I fragments between the *Bgl*II and *Sal*I sites of a *Pseudomonas* suicide replication plasmid (pGP704-L) to generate pVI681 and pVI682. The insert in plasmid pVI681 carries the first 573 bp of the *relA* coding sequence (codons 1 to 191), followed by the *Sma*I Km-3 cassette from p34S-Km3, the last 633 bp of the *relA* coding sequence, and the termination codon. The resulting gene replacement, designated *relA*::Km, thus has the internal 1,032 bp of KT2440 *relA* (encoding 344 amino acids of the predicted 746-residue protein) replaced by the Km^r gene. The insert in plasmid pVI682 carries 375 bp of the coding sequence of *spoT* (codons 5 to 129) followed by the *Sma*I Gm cassette from p34S-Gm, the last 372 bp of the *spoT* coding sequence, and the termination codon. The resulting gene replacement, designated *spoT*::Gm, thus has the internal 1,347 bp of KT2440 *spoT* (encoding 449 amino acids of the predicted 702-residue protein) replaced by the Gm^r gene. In both pVI681 and pVI682, the antibiotic resistance gene is orientated so it is transcribed in the same direction as *relA* and *spoT*, respectively.

Chromosomal insertions of *dmpR* and *xylR* into *P. putida* were achieved by cloning the fragment of interest onto pUTminiTn5-Km2 and pUTminiTn5-Tellurite and their subsequent transposition onto the *P. putida* chromosome, as previously described (60). The *xylR* gene inserted into *P. putida* KT2440 originated as an *Hpa*I fragment from pTK19, while the *dmpR* gene was obtained as a *Not*I fragment from pVI401. For both *dmpR* and *xylR*, KT2440 isolates derived from miniTn5-Km2 or miniTn5-Tellurite insertions promoted indistinguishable transcription from their cognate *luxAB* reporter plasmids (data not shown). The KT2440-*dmpR*-Tel and KT2440-*xylR*-Tel derivatives were chosen as the parents for generation of (p)ppGpp⁰ derivatives by replacement of the internal portions of *relA* and *spoT* by antibiotic resistance genes via homologous recombination as has been previously described in detail (50). The *relA*::Km derivatives were generated by conjugation of pVI681 from permissive replication host S17 λ pir on rich solid media. Tellurite and kanamycin were used for selection of first-site recombination, and second-site recombinants were detected by screening for sensitivity to the carbenicillin resistance marker of the donor plasmid. The presence of the mutant gene and absence of a wild-type copy were confirmed by PCR. The resulting strains were then used as the parents in an analogous second round of conjugation using pVI682 harboring *spoT*::Gm, with kanamycin and gentamicin used as selection for first-site recombination.

E. coli MG1655 and CF1693 derivatives with *lac* genes deleted were generated by P1 transduction to eliminate background β -galactosidase activity when the *lacZ* gene was to be used as the reporter. The Δ *lac* strains were analyzed for Po transcriptional profiles using the *luxAB* reporter and were found to be indistinguishable from their parent strains (data not shown).

Growth and culture conditions. All strains were grown at 30°C in Luria broth (LB) (57) unless otherwise indicated. Minimal M9 medium (57) was supplemented with 0.2% Casamino Acids, 10 mM glucose, and/or 100 μ g of thiamine/ml. Antibiotic concentrations used were as follows: carbenicillin, 100 μ g/ml for *E. coli* and 1 mg/ml for *P. putida*; gentamicin, 15 μ g/ml for *E. coli* and 100 μ g/ml for *P. putida*; kanamycin, 50 μ g/ml; tetracycline, 20 μ g/ml; potassium tellurite, 40 μ g/ml. Aromatic effectors (2-methylphenol for DmpR and 3-methylbenzyl alcohol for XylR) were added to final concentrations of 0.5 mM for *E. coli* and 2 mM for *P. putida*.

Before all assays and analyses, cultures were grown overnight in various media containing appropriate antibiotics for plasmid selection. To ensure balanced growth for reporter gene assays, cells from overnight cultures were diluted 1:1,000, grown to exponential phase, and then diluted once more before initiation of the experiment by addition of the relevant aromatics. For Western analyses, overnight cultures were also diluted 1:1,000, but, when they reached exponential phase, effectors were added and growth was allowed to continue for 4 to 5 h before the cells were harvested.

Reporter gene assays. Activity of the *lacZ* gene product β -galactosidase was determined spectrophotometrically, using *O*-nitrophenyl- β -D-galactopyranoside as the substrate, on cells permeabilized with chloroform and sodium dodecyl sulfate, as previously described (44). Luciferase assays of the *luxAB* gene product were performed on 100 μ l of whole cells using a 1:2,000 dilution of decanal, and light emission was measured by a PhL luminometer (Mediator Diagnostics). Data points for both assays are the averages of duplicate determinations, and the assay profiles over growth shown in the figures are representative of at least two independent experiments.

(p)ppGpp assays. Cells to be assayed for (p)ppGpp were grown in modified low-phosphate MOPS (morpholinepropanesulfonic acid) media (45) supplemented with 20 mM glucose and all 20 amino acids (69). Aliquots of the parent culture were taken at an optical density at 650 nm of $\sim$ 1.0 and were further grown in the presence of 100 μ Ci of ³²P (Amersham Pharmacia Biotech)/ml for 90 min. Cells were then collected by centrifugation, resuspended in ice-cold 1 M formic acid, and treated as described previously (5). Samples were spotted on

polyethyleneimine cellulose plates (Merck), fixed by methanol, and dried, and ascending chromatography in 1.5 M KH₂PO₄ buffer, pH 3.4, was used to separate the nucleotide species.

Western analyses of regulator expression levels. Crude extracts of cytosolic proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer to nitrocellulose filters, and Western blot analysis with polyclonal rabbit anti-DmpR antibodies raised against an A domain-truncated version of DmpR were as described previously (63). Antibody-decorated bands were revealed using Amersham Pharmacia Biotech chemiluminescence reagents as directed by the supplier. The amount of regulator proteins present was estimated by comparing 20 μ g of total cytosolic protein against a standard series (0.02- to 2-fmol loadings) of purified His-tagged DmpR (48) and XylR Δ A (from V. de Lorenzo) proteins. Several exposures of each series of experiments were performed so that quantification of samples relative to standards was always performed with exposures in the grey scale. The quantification shown in Table 2 makes the assumption that the anti-DmpR sera cross-react with full-length XylR with the same affinity as they do with the XylR Δ A standards.

RESULTS AND DISCUSSION

DmpR/Po and XylR/Pu *luxAB* and *lacZ* reporter gene transcriptional profiles are similar. Physiological regulation of both the *P. putida*-derived DmpR/Po and XylR/Pu minimal systems can be mimicked in *E. coli*, demonstrating that this heterologous host has functional counterparts to those that mediate global regulation in the native host. This made it reasonable to exploit available *E. coli* mutant strains in combination with minimal transcriptional reporter systems to identify the host-encoded factors that intercept these specific regulatory circuits (12, 68). In previous studies, the *Vibrio harveyi* luciferase *luxAB* gene has consistently been used as a reporter for the DmpR/Po system, whereas β -galactosidase encoded by *lacZ* has been used for the XylR/Pu system. While this allows trends and gross profiles between the two to be compared, questions of differential sensitivity, reporter half-lives, and other possibly unanticipated variables make finer comparison difficult. To overcome this limitation, corresponding *luxAB* and *lacZ* reporters were made for each of the DmpR/Po and XylR/Pu minimal regulatory units (Fig. 1).

Medium composition has a profound effect on transcription from the Po and Pu promoters. In defined minimal media, an immediate transcriptional response is observed from both promoters upon addition of aromatic effectors. However, addition of components of rich media results in a delay in transcription of several hours (40, 68, 69). In rich media such as LB, DmpR- and XylR-mediated transcription from their cognate promoters is not observed during exponential phase even when appropriate aromatic effectors are present. Transcriptional activities from these promoters only occur upon transition to stationary phase, where nutrients become limiting (12, 68). Since this comparative study utilizes both the native *P. putida* and *E. coli* mutant strains, *luxAB* and *lacZ* reporter systems were tested in both hosts grown on LB. As a starting point, we chose reporter systems previously observed to be capable of reproducing the physiological control of DmpR/Po. In *P. putida*, the test system consists of a monocopy chromosomal insertion of the regulator and an RK2-based plasmid bearing the promoter fused to the *luxAB* or *lacZ* reporter gene. In *E. coli*, the regulator and the promoter-reporter fusions are carried together on RSF1010-based plasmids. The strains were grown in LB in the presence of an aromatic effector specific to each regulator, and reporter activities were assayed over growth. From the data shown in Fig. 2 it can be seen first that

FIG. 2. Transcriptional profiles of DmpR/Po and XylR/Pu regulatory circuits in LB-grown *P. putida* (A, B, E, and F) and *E. coli* (C, D, G, and H), based on assays of luciferase (A to D) and β -galactosidase (β -gal; E to H) activities. Growth conditions are as stated in Materials and Methods. Solid symbols, log optical density at 600 nm (OD_{600}); open symbols, relative luciferase units (RLU) and Miller β -galactosidase units.

the genetic systems that exhibit physiologically responsive profiles for DmpR/Po also do so for XylR/Pu. Second, the transcriptional profiles obtained using *luxAB* (Fig. 2A to D) and *lacZ* (Fig. 2E to H) reporters are very similar. One minor difference is that a drop in transcriptional activity is more readily observed when using the *luxAB* reporter (e.g., compare Fig. 2C and G), probably due to the shorter half-lives of *luxAB* gene products. Nevertheless, the overall similarity indicates that the different reporters are unlikely to give dramatically different results. Therefore, in our comparative studies described below the *luxAB* reporters were routinely used, except in instances where a difference between the DmpR/Po and XylR/Pu physiological control had to be clearly established. In these cases, both reporters were used although only the *luxAB* data are presented.

Regulator expression levels in test strains. The impact of physiologically significant control mechanisms on specific regulatory circuits can frequently be masked or diminished if the regulator levels are markedly elevated over those found in the parental systems. Therefore it is important that reporter systems maintain the expression levels close to that found in the native regulatory circuit. In a comparative analysis such as that performed here it would be ideal if a single reporter system could be used in all experiments. However, the nonavailability of key mutants in *P. putida* requires the use of *E. coli* strains. The native promoter of *dmpR* functions poorly in *E. coli* (68), which is also evident from the different comparative efficiencies of the DmpR/Po and XylR/Pu systems in *P. putida* versus *E. coli* in Fig. 2. This consideration, in combination with the antibiotic resistance markers of plasmids carrying other genes

in *trans*, forced us to develop alternative genetic systems so that expression levels more closely reflecting those of the native system could be achieved. All the test systems used here were quantified for the levels of DmpR and XylR by Western analysis, and these levels were compared to the native expression levels from pVI150 and pWW0. As shown in Table 2, the relative expression of XylR is approximately 10-fold higher than that of DmpR when the two proteins are expressed from their native plasmids in the parental hosts. Importantly, with the *P. putida* monocopy systems, expression levels do not exceed 2.5 times the native level. The *E. coli* test systems have an overall two- to fourfold reduction in regulator expression compared to those of *P. putida*, despite being present on a 16-copy plasmid. Minor difference in regulator levels between *E. coli* mutants and their isogenic parents can be seen but are too small to account for the dramatic trends shown in the experiments described below.

FtsH is not universally required for σ^{54} -dependent transcription. The levels of *E. coli* ATP-dependent FtsH protease are controlled in response to physiological cues. This protease is an essential factor for aerobic survival of *E. coli*, with *ftsH* mutations requiring an additional suppressor mutation in order for the bacteria to grow (55). In an FtsH⁻ *E. coli* strain, XylR-mediated transcription from Pu is drastically reduced, as was transcription mediated by three other σ^{54} -dependent regulators tested, NtrC, NifA, and PspF (8). This finding spurred the idea that FtsH is an essential element for σ^{54} transcription, at least in *E. coli* (8). A search of the as yet unfinished *P. putida* KT2440 genomic database shows that there is an FtsH homologue (62% identity; data not shown) which may play a

TABLE 2. Regulator expression levels

Strain	DmpR test system	DmpR level (pmol/mg of CE ^a)	XylR test system	XylR level of (pmol/mg of CE)	XylR/DmpR ratio
Native strain	<i>Pseudomonas</i> sp. strain CF600	4.4	KT2440 (pWW0::Tn401)	45.5	10.3
<i>P. putida</i>	KT2440:: <i>dmpR</i> ^b	9.8	KT2440:: <i>xylR</i> ^b	29.3	3.0
	KT2440:: <i>dmpR</i> -Po- <i>luxAB</i>	4.4	KT2440:: <i>xylR</i> -Pu- <i>luxAB</i>	26.0	5.9
<i>E. coli</i>					
MG1655 Δ lac	pV1466 <i>dmpR</i> -Po- <i>luxAB</i>	1.0	pV1677 <i>xylR</i> -Pu- <i>luxAB</i>	19.5	19.5
	pV1679 <i>dmpR</i> -Pu- <i>luxAB</i>	1.0	pV1678 <i>xylR</i> -Po- <i>luxAB</i>	19.5	19.5
CF1693 Δ lac	pV1466	1.5	pV1677	13.0	10.6
	pV1679	1.5	pV1678	19.5	13.0
A8514	pV1466	2.0	pV1677	39.0	19.5
	pV1679	2.0	pV1678	42.3	21.2
A8926	pV1466	2.4	pV1677	16.3	6.8
	pV1679	2.4	pV1678	19.5	8.1

^a CE, crude extract.^b No differences in DmpR and XylR levels in KT2440 derivatives harboring chromosomal copies of *dmpR* or *xylR* delivered via miniTn5-Km2 or miniTn5-Tellurite were found. Likewise no differences in DmpR and XylR levels between the (p)ppGpp⁰ derivatives (PP1::*dmpR* and PP2::*xylR*) and the parent strains were observed.

similar role in this host. Despite intensive efforts we were unable to generate a *P. putida* FtsH⁻ mutant by a method that has proven productive for other host genes (see below), probably due to the essential nature of FtsH in *P. putida*. Therefore, we employed the same *E. coli* FtsH⁻ mutant and parental strains as Carmona and coworkers (8) to test the effect of FtsH on the DmpR/Po circuit. As shown in Fig. 3A and B, while the XylR/Pu system is clearly defective in the absence of FtsH, the DmpR/Po system is fully functional. In addition, exponential-phase silencing of Po is somewhat diminished in the FtsH⁻ mutant (Fig. 3B). Therefore, FtsH is not required for DmpR/Po

transcription and is clearly not an essential factor for σ^{54} -dependent transcription per se.

The observation that overexpression of FtsH abolishes exponential silencing of the XylR/Pu system in rich media, in conjunction with the findings that the defective transcription from Pu in the absence of FtsH can be partially rescued by σ^{54} overexpression, suggested a possible link to E σ^{54} availability and/or activity (8). While σ^{54} levels were not observed to differ from that of the parent in the FtsH⁻ strain, one speculation, partly based on the fact that FtsH is involved in controlling turnover of the heat shock σ^{32} factor (4), was that FtsH may also directly or indirectly control σ^{54} turnover or activity (8). If this were the case, then the Pu transcription-defective phenotype of the FtsH⁻ mutant strain would imply that the mutant contains less available E σ^{54} than its parent. Following this argument, the drastically different effects of FtsH on the XylR/Pu and DmpR/Po activity would have to be attributed to different promoter affinities for E σ^{54} and the phenomenon of FtsH dependence should accompany the promoter. Available *in vitro* data suggest that while the affinities of Po and Pu for E σ^{54} are similar in the presence of IHF, Po has a significantly higher apparent affinity than Pu in the absence of IHF (9, 67). Therefore, to test the contribution of promoter versus regulator to FtsH sensitivity, we performed regulator-swapping experiments in which the effects of FtsH on DmpR-mediated transcription of Pu and XylR-mediated transcription from Po were measured. As shown in Fig. 3C and D the data clearly demonstrate that the regulator, rather than the promoter, determines sensitivity to the absence of FtsH.

Western analysis of the levels of DmpR and XylR in the FtsH mutant strain indicate that DmpR levels are comparable to those found in the parent strain, while XylR levels are decreased approximately twofold (Table 2). However, the reduced levels of XylR are similar or higher than those found in other *E. coli* backgrounds (e.g., MG1655 Δ lac and CF1693 Δ lac) where XylR-mediated transcription is readily observed. While one cannot fully rule out the possibility that, for unknown reasons, higher threshold levels of XylR may be required to promote transcription in the A8514 *E. coli* lineage, the data

FIG. 3. Luciferase transcriptional profiles of Po and Pu promoters activated by their native regulators (A and B) or nonnative regulators (C and D) in *E. coli* A8514 (FtsH⁺) and A8926 (FtsH⁻). Solid symbols, culture growth; open symbols, luciferase response. Note that, as has been found previously, the absolute values of reporters for different *E. coli* strains vary (68) and that transcriptional levels are higher in this lineage of *E. coli* than in the MG1655 lineage used in Fig. 2. RLU, relative luciferase units; OD₆₀₀, optical density at 600 nm.

described above suggest that the effect of FtsH is channeled through some differentiating property of XylR and DmpR other than expression levels. Circumstantial evidence suggested that, besides being a protease, FtsH can act as a chaperone (reviewed in reference 59). Indeed, multiple sequence alignment combined with advanced computer methods for iterative databases placed FtsH in the AAA⁺ superfamily of chaperone-like ATPases. This family of proteins often act like “molecular machines,” performing functions that assist in assembly, operation, or disassembly of protein complexes, and σ^{54} -dependent activators themselves fall into a subgroup within this superfamily (46). Where tested, σ^{54} -dependent regulators have been found to oligomerize from lower- to higher-order multimeric states in response to signal reception and nucleotide binding by the central C domain (e.g., NtrC [27, 43] and XylR [51]). This is also the case for DmpR; however, unlike XylR and other σ^{54} regulators, DmpR readily undergoes oligomerization in the absence of DNA binding (71). This raises the intriguing possibility that for the FtsH-dependent σ^{54} regulators, FtsH may be required for correct folding or for proper assembly and/or disassembly of regulator multimers or regulator- σ^{54} complexes.

PtsN mediates glucose repression of the XylR/Pu system but not the DmpR/Po system. XylR/Pu is subject to an additional level of physiological regulation, in which the presence of glucose reduces the level of transcription from Pu by two- to threefold. Work with the native *P. putida* host has previously shown that this level of regulation involves the *ptsN* gene, which encodes the IIA^{Ntr} component for an alternative PTS, located in a gene cluster downstream of *rpoN* (11, 14). To test if this level of control is manifested in the DmpR/Po regulatory circuit, we employed the same *P. putida* KT2440 background. To maintain the same growth rate independent of the presence or absence of glucose, we also used the same medium conditions as Cases and colleagues (14), namely, M9 minimal media supplemented with casein amino acids in the presence and absence of glucose. *P. putida* reporter strains consisting of a monocopy of the regulator gene on the chromosome and RK2-based promoter-*luxAB* reporters were used to monitor DmpR/Po and XylR/Pu transcription. Promoter activities were monitored over growth, and the values shown in Fig. 4 are those taken at postexponential phase, where the activities were observed to be in their peaks. Note that growth in these media results in three- to fivefold-higher promoter activities than growth in LB (Fig. 2A and B). The data in Fig. 4 show that no effect of glucose is imposed on the DmpR/Po system while a twofold effect, as observed previously, was found with the analogous XylR/Pu reporter. To ascertain that this was not a result of the rather low XylR/DmpR expression ratio of the test system (Table 2), the experiment was repeated with the other *P. putida luxAB* reporter systems available, i.e., the KT2440::*dmpR*-Po-*luxAB* and KT2440::*xylR*-Pu-*luxAB* strains, with similar results (data not shown).

Regulator-swapping experiments demonstrate that the susceptibility to glucose repression depends on the identity of the regulators (Fig. 4). We infer from this that, like the action of FtsH, the mechanism by which PtsN functions is channeled through the activity of the regulator rather than the inherent properties of the promoter. The function of the *P. putida ptsN*-encoded IIA^{Ntr} enzyme has not yet been elucidated (14).

FIG. 4. Luciferase activity of *P. putida* KT2440::*dmpR*-Km and KT2440::*xylR*-Km carrying RK2-based plasmid pVI673 (Po-*luxAB*) or pVI674 (Pu-*luxAB*). Cells were grown in M9 media with 0.2% casein amino acids (CAA) and the appropriate aromatic effector and further supplemented with 10 mM glucose where indicated. Peak activities shown are averages of triplicate determinations from two independent experiments. RLU, relative luciferase activity.

However, PTSs have regulatory roles besides phosphotransfer of sugars across the membrane, e.g., generation of signals for catabolite repression, nitrogen metabolism, and chemotaxis (56, 66). Two-dimensional gel electrophoresis of a *P. putida ptsN* mutant shows that PtsN has global effects on protein expression and that these effects do not affect all σ^{54} -dependent promoters and the effects are not limited to σ^{54} -dependent genes (13). Whatever the precise mechanism by which the *P. putida* glucose-PtsN link impacts on XylR, it clearly does not intercept DmpR. Hence, like FtsH, PtsN represents a regulatory factor that impacts the XylR/Pu system but not the DmpR/Po system and mediates its effect via the regulator rather than the promoter.

(p)ppGpp⁰ *E. coli* and *P. putida* differ in prototrophy. Production of the signaling molecule (p)ppGpp is a herald of metabolic stress. In *E. coli* the ribosome-bound RelA (p)ppGpp synthetase catalyzes the production of (p)ppGpp from GTP when activated by the arrival of an uncharged tRNA on the ribosome, while SpoT (which is primarily responsible for [p]ppGpp degradation), catalyzes (p)ppGpp synthesis in response to glucose starvation (reviewed in reference 15). The binding of (p)ppGpp to RNA polymerase results in a reorganization of the global transcriptional patterns in response to the changing growth conditions primarily by down-regulating transcription from stringent promoters (reviewed in references 15 and 16). A role for (p)ppGpp in up-regulation of transcription from Po and as the metabolic link between host physiology and the DmpR/Po regulatory system was first indicated by the finding that transcription of the DmpR/Po system was dependent on growth conditions that elicit high (p)ppGpp levels. Further evidence came from the findings that gratuitous synthesis of (p)ppGpp under normally nonpermissive conditions abolished exponential silencing in *P. putida* and that the drastically reduced transcription from Po in a (p)ppGpp⁰ *E. coli* strain could be rescued by mutations within the transcriptional apparatus (69). Similar gratuitous production of (p)ppGpp, monitored using a *lacZ* reporter, likewise abolished exponential silencing of the XylR/Pu system in LB. However, in a (p)ppGpp⁰ *E. coli*

FIG. 5. Chromatographic analysis of ^{32}P -labeled nucleotides of the indicated *E. coli* and *P. putida* strains as described in Materials and Methods. The abilities of the strains to grow on M9 minimal medium plates supplemented with 10 mM glucose and 100 μg of thiamine/ml are indicated (bottom).

background, the reduction in transcriptional activity was minimal (10).

To assess the dependence of the two systems on (p)ppGpp in their native *P. putida* host, we generated a (p)ppGpp 0 strain of KT2440. With *E. coli* RelA and SpoT, a search of the as yet unfinished *P. putida* KT2440 genomic database identified a single RelA homologue (47% identity, 68% similarity) and a single SpoT homologue (54% identity, 72% similarity) in this strain. As shown in Fig. 5, like its *E. coli* counterpart, the *P. putida* double mutant is a full (p)ppGpp 0 strain, suggesting that no other (p)ppGpp synthetase exists in KT2440. However, unlike their *E. coli* (p)ppGpp 0 counterpart, the (p)ppGpp 0 *P. putida* strains are prototrophic, being able to grow on minimal media lacking amino acids (Fig. 5). Both the *E. coli* and *P. putida* double mutants were generated by similar gene replacement strategies in which the internal portions of the *relA* and *spoT* genes are replaced by antibiotic resistance genes (see Materials and Methods). Prototrophy of *E. coli* appears to be in fine balance since single amino acid substitutions of RNA polymerase that restore prototrophy to (p)ppGpp 0 strains can readily be selected on minimal media (15, 33). Our *P. putida* strain construction strategy avoided growth on anything but rich media. Moreover, >10 independent (p)ppGpp 0 *P. putida* isolates have been tested both with respect to prototrophy and their expression profiles by using a Po-*luxAB* reporter. All derivatives exhibit the same behavior; thus we conclude that the difference in the ability to grow on minimal media in the absence of amino acids is a genuine difference between (p)ppGpp 0 derivatives of *E. coli* and *P. putida*. Given the above and the fact that the amino acid sequences of the components of the transcriptional apparatus of *E. coli* and *P. putida* are divergent (examples of identities: RpoB, 74%; RpoC, 75%; RpoD, 66%; RpoN, 56%; data not shown), a difference in prototrophy is not surprising and probably reflects differences in the abundance and/or properties of the RNA polymerases.

Influence of (p)ppGpp on the DmpR/Po and XylR/Pu circuits in *E. coli* and *P. putida*. Luciferase reporter plasmids were introduced into (p)ppGpp $^+$ *E. coli* and *P. putida* strains and their otherwise isogenic (p)ppGpp 0 counterparts. For *E. coli*, the reporter systems consisted of RSF1010-based plasmids car-

rying both the regulator and promoter-*luxAB* fusion, while for *P. putida*, the regulators were at monocopy on the chromosome, with the promoter-*luxAB* fusions carried on RSF1010-based plasmids. In *E. coli*, DmpR-mediated regulation of Po transcription decreases by approximately 10-fold (Fig. 6A) while XylR-mediated Pu transcription is only marginally affected, with maximum reduction of a mere 30 to 50% (Fig. 6B). These results reaffirm the marginal dependence of the XylR/Pu circuit on (p)ppGpp, in marked contrast to the strong dependence of the DmpR/Po circuit in *E. coli*. However, a somewhat different result was found with *P. putida*. In the native host, the DmpR/Po system is still strongly dependent on (p)ppGpp (Fig. 6C), although to a lesser extent than in *E. coli*. For XylR/Pu, the expression profiles in the ppGpp $^+$ and ppGpp 0 strains both still exhibit an abrupt shift in expression at the transition from exponential to stationary phase, but with the maximum level achieved in the ppGpp 0 strain reduced to approximately that observed with the ppGpp $^+$ strain after prolonged incubation (Fig. 6D). Thus while the basic trends and main conclusions drawn from the work in *E. coli* are verified in *P. putida*, the magnitude of dependence of the two systems in the different hosts varies.

The above finding raises the question of what factors determine the somewhat different responses in the two hosts. Work with *E. coli* demonstrated that lack of (p)ppGpp does not greatly affect the levels of DmpR or σ^{54} (69). Western analysis comparing DmpR and XylR levels in the *E. coli* and *P. putida* (p)ppGpp $^+$ and (p)ppGpp 0 strains did not reveal any detectable differences between the parents and mutant derivatives (Table 2). Therefore, expression levels of these key players are unlikely to explain the differences observed with reporters in *E. coli* and *P. putida*.

Binding of (p)ppGpp to the transcriptional apparatus is believed to occur through a site at an interface of the β - and β' -subunit (RpoB, RpoC), with binding affecting multiple steps in transcription from specific promoters (reviewed in references 15 and 16). One major consequence of the resulting negative regulation of stringent promoters is an increase in the amount of limiting core RNA polymerase. A growing body of genetic evidence has suggested that (p)ppGpp binding may also influence the outcome of sigma factor competition for the available core enzyme (26, 33, 38, 69) and may thereby alter the amount of holoenzyme associated with alternative sigma factors. Hence differences in the amount of available $\text{E}\sigma^{54}$ may in part be responsible for the higher Po output observed in (p)ppGpp 0 *P. putida*.

In addition to direct effects on promoter kinetics, accumulation of ppGpp in vivo can also have major indirect effects on promoter activity due to its influence on transcription of global regulatory factors such as σ^s and IHF (3, 32). IHF is needed for optimal promoter output from both the DmpR/Po and XylR/Pu systems (9, 67). For DmpR/Po, the system is equally dependent on IHF in both *E. coli* and *P. putida*, and coexpression experiments have demonstrated that additional IHF does not relieve the dramatic reduction in transcription from Po in (p)ppGpp 0 *E. coli* (67, 69). However, the XylR/Pu system is much more dependent on IHF in *P. putida* than in *E. coli* (6). Hence, it is possible that indirect effects on IHF levels may play a role in the reduction of Pu output in (p)ppGpp 0 *P. putida*.

FIG. 6. Luciferase transcriptional profiles of Po and Pu promoters activated by their native regulators (A to D) or nonnative regulators (E to H), in isogenic *E. coli* (A, B, E, and F) and *P. putida* (C, D, G, and H) (p)ppGpp⁺ and (p)ppGpp⁰ strains. *E. coli* (p)ppGpp⁺ MG1655Δlac and (p)ppGpp⁰ CF1693Δlac strains and *P. putida* (p)ppGpp⁺ KT2440-dmpR-Tel and -xylR-Tel and (p)ppGpp⁰ PP1-dmpR and -PP1-xylR strains were used. Solid symbols, culture growth; open symbols, luciferase response. RLU, relative luciferase units; OD₆₀₀, optical density at 600 nm.

Role of the promoter versus regulator in sensitivity to the absence of (p)ppGpp. To see if the difference in the influence of (p)ppGpp on the DmpR/Po and XylR/Pu systems could be ascribed to regulator or promoter properties, regulator-swapping experiments were performed with both hosts. The data show that, in the *E. coli* (p)ppGpp⁰ background, DmpR-driven Pu transcription is reduced by about fivefold (Fig. 6E) while XylR-driven transcription from Po was reduced three- to fourfold (Fig. 6F). Thus, having either component of the DmpR/Po pair renders the system more dependent on (p)ppGpp, although not to the same extent (10-fold) as for the original pair. In *P. putida*, on the other hand, the nature of the regulator is the predominant component that determines the transcriptional outcome, since profiles with the cognate and noncognate promoters are very similar (Fig. 6C, D, G, and H). Hence, in both *E. coli* and *P. putida*, the nature of the regulator plays a dominant role in the sensitivity to the absence of (p)ppGpp. The simplest interpretation of this finding is that ppGpp binding to Eσ⁵⁴, in addition to its other effects, also modulates the efficiency with which DmpR interacts with Eσ⁵⁴ to relieve the repressive effect of σ⁵⁴ on core polymerase activity.

The multitude of effects of the (p)ppGpp signaling molecule in vivo makes it difficult to put quantitative levels on the contribution of the different regulatory mechanisms of (p)ppGpp to physiological coupling. Nevertheless, the finding that mutations in the transcriptional apparatus can restore transcription to the DmpR/Po circuit provides strong support for direct effects of (p)ppGpp on Po promoter kinetics (69). Further support for this conclusion has been provided by the recent

demonstration of a four- to sixfold ppGpp-mediated stimulation of in vitro transcription from Po by an N-terminally truncated derivative of XylR (10). In addition to showing direct effects on promoter kinetics, previous work has provided genetic evidence for a role for (p)ppGpp in altering sigma factor competition in favor of σ⁵⁴, which may also contribute to the rapid transition and higher net transcriptional output of Po in (p)ppGpp⁺ strains (Fig. 6) (69). The data in Fig. 6 add a third potential influence of (p)ppGpp on metabolic coupling by providing suggestive evidence for a role for (p)ppGpp in modulating the conformation of the transcriptional apparatus to allow efficient interaction with DmpR. The contribution of (p)ppGpp to each of these processes in vitro certainly warrants more attention and is the focus of our future research.

Concluding remarks. The work presented here demonstrates that DmpR/Po and XylR/Pu regulatory circuits, two mechanistically and functionally similar σ⁵⁴ regulator/promoter pairs, are integrated into the global regulatory network through different host factors. Neither of the two factors identified to impact the XylR/Pu system (FtsH and PtsN) influences the DmpR/Po system, while (p)ppGpp impacts the DmpR/Po system to a much greater extent than the XylR/Pu system. The comparative analysis presented here illustrates that host-imposed regulation of these systems predominantly exploits properties of the specific regulator to exert dominant regulatory control over the specific regulatory circuits. However, despite the integration of different global factors, the biological outcome is functionally analogous: a transcriptional response adapted to initiate aromatic degradation only in the

absence of other preferred nutrients. This is interesting not only from the mechanistic but also the evolutionary point of view. Sequence comparison of aromatic catabolic operons reveals a "patchwork" assembly pattern suggesting evolutionary recruitment of simple metabolic modules to generate catabolic pathways (reviewed in reference 72). The acquisition of specific regulatory genes has likewise been proposed to occur in a patchwork manner involving recruitment of regulators that happen to have some appropriate signal response property that can be further refined through selection (21). This work suggests that integration of dominant global regulatory circuits can similarly occur in a patchwork manner in which global regulatory factors latch onto different properties of the specific regulatory circuit. It is possible that the interconnecting and overlapping nature of global regulatory networks bypasses the need for specific regulatory units to be woven into the tapestry of cellular physiology at one specific point in order to achieve analogous biological output.

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